GEORGIA INSTITUTE OF TECHNOLOGY — OFFICE OF CONTRACT ADMINISTRATION

PROJECT ADMINISTRATION DATA SHEET

[X] ORIGINAL [ ] REVISION NO. ____

Project No. G 33-605 NIH — DATE 9-21-82

Project Director: Dr J.C. Powers — School/~~Lab~~ Chemistry

Sponsor: DHHS/PHS/NIH - National Heart, Lung, and Blood Institute

Type Agreement: Grant No. 5-R01-HL 22530-05

Award Period: From 8-1-82 To 7-31-83 (Performance) 10-31-83 (Reports)

Sponsor Amount: $ 58,653 — Contracted through: ~~GTRI~~/GIT

Cost Sharing: $ 3,087 (G33-339)

Title: Active Site Studies On Blood Proteases

ADMINISTRATIVE DATA — OCA Contact Don Hasty

1) Sponsor Technical Contact:

Dr Alan Levine
Division of Blood Diseases and Resources
National Heart, Lung + Blood Institute
Bethesda, MD 20205
Phone (301) 496-5911

2) Sponsor Admin/Contractual Matters:

Miss Margaret Heedrick
Grants Operation Branch
Division of Extramural Affairs
National Heart, Lung + Blood Institute
Bethesda, MD 20205

Defense Priority Rating: N/A — Security Classification: N/A

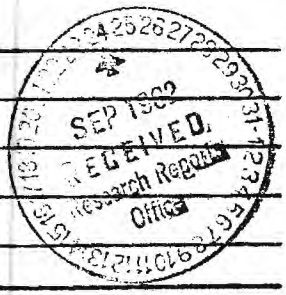
RESTRICTIONS

See Attached NIH Supplemental Information Sheet for Additional Requirements.

Travel: Foreign travel must have prior approval — Contact OCA in each case. Domestic travel requires sponsor approval where total will exceed greater of $500 or 125% of approved proposal budget category.

Equipment: Title vests with N/A - None proposed -

COMMENTS: Follow On to G33-604



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FORM OCA 4:781

GEORGIA INSTITUTE OF TECHNOLOGY OFFICE OF CONTRACT ADMINISTRATION

SPONSORED PROJECT TERMINATION SHEET

SR 3 84

Date September 20,1983

Project Title: Active Site Studies On Blood Proteases

Project No: G-33-L05

Project Director: Dr. James C. Powers

Sponsor: DHHS/PHS/NIH- National Heart,Lung, & Blood Institute.

Effective Termination Date: 7/31/83

Clearance of Accounting Charges: _____

Grant/Contract Closeout Actions Remaining:

- ☐ Final Invoice and Closing Documents
- ☐ Final Fiscal Report
- ☐ Final Report of Inventions
- ☐ Govt. Property Inventory & Related Certificate
- ☐ Classified Material Certificate
- ☒ Other Annual Report of Expenditures

*NOTE:Continued by G-33-L06

Assigned to: Chem. (School/Laboratory)

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GTRI

FORM OCA 10:781

G-33-L05

SECTION IV PROGRESS REPORT SUMMARY	GRANT NUMBER HL 22530-06	
PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR	PERIOD COVERED BY THIS REPORT	
POWERS, JAMES C.	FROM	THROUGH
NAME OF ORGANIZATION GEORGIA INSTITUTE OF TECHNOLOGY	5/28/82	5/14/83

TITLE *(Repeat title shown in item 1 on first page)*

ACTIVE SITE STUDIES ON BLOOD PROTEASES

(SEE INSTRUCTIONS)

Publications

"Reactivity of Bovine Blood Coagulation Factor IXa, Factor Xa, and Factor XIa toward Fluorogenic Peptides Containing the Activation Site Sequences of Bovine Factor IX and Factor X," Castillo, M.J., Kurachi, K., Nishino, N., Ohkubo, I., and Powers, J.C.(1983) Biochemistry 22 , 1021-1029.

Publications Submitted

"Benzyl-4-guanidinothiobenzoate Hydrochloride: A New Active-Site Titrant for Trypsin-like Enzymes," Cook, R.R., and Powers, J.C.(1983) Biochem. J. , submitted.

"Mammalian Trypsin-like Enzymes. Comparative Reactivities of Human Skin Tryptase, Human Lung Tryptase and Bovine Trypsin with Peptide 4-Nitroanilide and Thioester Substrates," Tanaka, T., McRae, B.J., Cho, K, Cook, R., Fraki, J.E., Johnson, D.A., and Powers, J.C.(1983) J. Biol. Chem. , submitted.

"Active Site Mapping of Blood Coagulation Serine Protease. Studies of the S3 Subsite of Factor IXa, Factor Xa, Factor XIa, Factor XIIa, Thrombin and Activated Protein C Using Tripeptide 4-Nitroanilide Substrates and the S2 and S1' Subsite of Protein C and Factor XIIa Using Amino Acid and Dipeptide Thioesters. Comparison of the Relative Reactivities of Human and Bovine Factor XIIa and Activated Protein C," Cho, K., Tanaka, T., Cook, R.R., Kisiel, W., Fujikawa, K., Kurachi, K., and Powers, J.C.(1983) Biochemistry , submitted.

Progress Report

Scientific Goals. The goal of this research is to understand the nature of the active sites of plasma serine proteases and other related trypsin-like enzymes. These proteins are the basis of important physiological processes such as blood clotting, fibrinolysis and the immune defense mechanism involving the complement system. The tools for the investigation will be synthetic peptides. Synthetic peptides corresponding to sites which are cleaved by various plasma proteases will be prepared. The binding of these peptides to plasma proteases and their rates of hydrolysis will be determined. In addition we plan to design and synthesize small peptide thioesters which are more reactive toward serine proteases than simple peptides. These will be utilized to develop more sensitive and specific assays for individual enzymes.

Progress. We have synthesized peptides containing the activation sequences of bovine factors IX and X and studied their reaction with bovine factors XIa and IXa. The substrates contain a fluorophore and a quenching group which are separated upon enzymatic hydrolysis with a resultant increase in fluorescence which was utilized to measure hydrolysis rates. Factor XIa cleaved all the peptides bearing factor IX activation site sequences. The kinetic behavior of factor XIa toward the synthetic peptide substrates indicates that it has a minimal extended substrate recognition site at least five residues long and has favorable interactions over seven subsites. The hexapeptide Abz-Glu-Phe-Ser-Arg-Val-Val-Nba was the most specific factor XIa substrate and was not hydrolyzed by factors IXa or Xa or thrombin. Factor IXa failed to hydrolyze any of the synthetic peptides bearing the activation site sequence of factor X. This enzyme slowly cleaved four peptide substrates with factor IX activation site sequences. Thrombin failed to cleave any of the peptides examined. Both factor IXa and XIa cleaved the peptide substrates at similar rates to their natural substrates under comparable

conditions. However the rates were substantially lower than optimum activation rates observed in the presence of calcium, phospholipids, and protein cofactors. This work has been published.

Benzyl-4-guanidinothiobenzoate hydrochloride has been synthesized and demostrated to be useful for active-site titration of trypsin, thrombin, human lung tryptase, activated protein C, factor XIIa and factor Xa. The titration is based upon rapid formation of a stable acyl enzyme with stoichiometric release of benzyl thiol. The thiol production is quanititated by including 4,4'-dithiodipyridine in the reaction mixture and measuring the increase in absorbance at 324 nm. Advantages of this reagent as a titrant include: flexibility in detection of the released thiol, selectivity between trypsin and chymotrypsin-like enzymes, relative stablity of the reagent under titration conditions, and high sensitivity. The reagent should prove useful as an alternate to 4-nitrophenyl-4-guanidinobenzoate hydrochloride for the determination of active site concentrations of trypsin-like enzymes.

The subsite specificity of human lung and skin tryptase (trypsin-like enzyme) has been studied at pH 7.5 using 17 amino acid and dipeptide thioester substrates and 14 tripeptide 4-nitroanilide substrates. The reactivity and specificity of the human tryptases were compared with bovine trypsin and other trypsin-like enzymes. Neither tryptase was similar to either kallikrein or factor XIIa(Hageman Factor). The skin enzyme was the most reactive as measured by the specificity constant. The best substrate was Z-Lys-Arg-SBu-i which had a k_{cat}/K_M value of 59,000,000 $M^{-1}s^{-1}$. Both enzymes have extended substrate binding sites and proline residues at P_3 substantially decrease k_{cat}/K_M. Both enzymes preferred the tripeptide 4-nitroanilides with a P_2 Gly residue over Phe, and both favored the P_3 substrate Z-Lys-Gly-Arg-NA over similar substrates containing 6 other representative amino acid residues at P_3. The lung enzyme was inhibited over three times faster by p-amidinophenylmethane sulfonyl fluoride. The preference of the skin trypstase for substrates with two terminal basic residues indicates that this enzyme could process prohormones and proproteins which contain this structural feature at the cleavage site. The substrates should be useful for the further characterization of the physiologic function of tryptases.

A series of fourteen tripeptide 4-nitroanilide substrates of the type Z-AA-Gly-Arg-NA and Z-AA-Phe-Arg-NA where AA = Ala, Asn, Glu, Lys, Phe, Pro, or Ser were used to map the S_3 subsite of blood coagulation serine proteases. The enzymes studied include thrombin, factor IXa, factor Xa, factor XIa, human factor XIIa, and bovine and human activated protein C. Kinetic constants for the enzymatic hydrolysis of the substrates by each enzyme were determined and used to compare the relative reactivities of the individual enzymes. Human factor XIIa showed the highest reactivity of all the coagulation proteases studied and was also very substrate specific. The best substrate was Z-Lys-Phe-Arg-NA. Bovine activated protein C (best substrate = Z-Ser-Phe-Arg-NA), factor Xa (best substrate = Z-Glu-Gly-Arg-NA) and thrombin (best substrate = Z-Lys-Gly-Arg-NA) were the group of enzymes that showed next highest reactivity toward the substrates. Bovine protein C and factor Xa displayed relatively little substrate specificity. Thrombin, likewise, demonstrated relatively little specificity but strongly disliked Z-Pro-Phe-Arg-NA which was not hydrolyzed at all. Human activated protein C (best substrate = Z-Ser-Phe-NA) and factor XIa (best substrate Z-Glu-Gly-Arg-NA) are moderately reactive enzymes. Human activated protein C is an extremely specific enzyme. Factor XIa, although very slow, hydrolyzed ten of the fourteen substrates. Human factor XIIa (best substrate = Z-Phe-Arg-SBu-i) and both bovine (best substrate = Z-Arg-SBzl) and human activated protein C (Z-Phe-Arg-SBu-i) were studied with a set of amino acid and dipeptide thioester substrates. The best thioester substrates were 10-359 fold better substrates than the best 4-nitroanilide substrates. Comparison of bovine and human factor XIIa, and bovine and human activated protein C showed substantial differences both in reactivity and in subsite preferences.

There were also significant similarities. These substrates should be useful for the future development of sensitive and specific assays for coagulation enzymes.

Specific Objectives for Next Year. One objective for the coming year will be to synthesize peptides containing the structural features of Gla residues. Possible structures would include $(HO_2C)_2CHCH_2CH_2CO$-Spacer-peptide. Our purpose is to design peptides which could bind to phospholipid vesicles in the same manner as the natural substrates for factory IXa and Xa. Such substrates might be significantly more reactive and specific than the ones we have prepared to date. One of our major overall goals for the future is to increase the specificity of various substrates.

We also intend to increase the sensitivity of some of our thioester substrates. We plan to incorporate a fluorophor and a quenching group in a substrate with a thioester bond. As the substrate is cleaved the fluorescence will increase and the activity of the enzyme can be followed kinetically. Due to the high reactivity of the thioester bond and high sensitivity of fluorescence detection, we hope to increase the sensitivity of our assays by several fold.

Another objective will be to investigate the reaction of thioesters with zymogens. We have synthesized Boc-Ala-Ala-AA-SBzl where AA is 15 different amino acid residues. We plan to study these substrates with zymogens such as trypsinogen, chymotrypsinogen, factor X and prothrombin. If these studies are successful, we may try to do some subsite mapping of the zymogens. These studies could lead to direct assays for coagulaltion factors without the need of prior proteolytic activation.

Finally, we are doing pattern recognition studies with the blood coagulation enzymes. We have now measured kinetic constants for the hydrolysis of 30 different substrates by over 14 trypsin-like enzymes. We are now trying to relate the various enzymes by their reactivity toward the various substrates.